Brazilian Journal of Biology®

ISSN 1519-6984 (Print)
ISSN 1678-4375 (Online)

Original Article

Protease production and molecular characterization of a protease dipeptidyl-aminopeptidase gene from different strains of *Sordaria fimicola*

Produção de protease e caracterização molecular de um gene de protease – DPAP (dipeptidil-aminopeptidase) de diferentes cepas de *Sordaria fimicola*

U. Naureen[a]*, A. Kayani[b], F. Akram[a], A. Rasheed[a] and M. Saleem[a]

[a]University of the Punjab, Department of Botany, Molecular Genetics Research Laboratory, Lahore, Pakistan
[b]Government Model Degree College for Women, Model Town, Lahore, Pakistan

Abstract

The current research was designed to reach extracellular protease production potential in different strains of *Sordaria fimicola* which were previously obtained from Dr. Lamb (Imperial College, London) from North Facing Slope and South Facing Slope of Evolution Canyon. After initial and secondary screening, two hyper-producers strains S2 and N6 were selected for submerged fermentation and cultural conditions including temperature, pH, incubation period, inoculum size, substrate concentration, and different carbon and nitrogen sources were optimized for enzyme production. S2 strain showed maximum protease production of 3.291 U/mL after 14 days of incubation at 30 °C with 7 pH, 1% substrate concentration and 1 mL inoculum, While N6 strain showed maximum protease production of 1.929 U/mL under fermentation optimized conditions. Another aim of the present research was to underpin the biodiversity of genetics and post-translational modifications (PTMs) of protease DPAP (peptidyl-aminopeptidase) in *Sordaria fimicola*. Five polymorphic sites were observed in amino acid sequence of *S. fimicola* strains with reference to *Neurospora crassa*. PTMs prediction from bioinformatics tools predicted 38 phosphorylation sites on serine residues for protease peptidyl-aminopeptidase in S1 strain of *S. fimicola* while 45 phosphorylation sites on serine in N7 strain and 47 serine phosphorylation modifications were predicted in *N. crassa*. Current research gave an insight that change in genetic makeup effected PTMs which ultimately affected the production of protease enzyme in different strains of same organism (*S. fimicola*). The production and molecular data of the research revealed that environmental stress has strong effects on the specific genes through mutations which may cause genetic diversity. *S. fimicola* is non- pathogenic fungus and has a short life cycle. This fungus can be chosen to produce protease enzyme on a commercial scale.

Keywords: enzyme production and optimization, fungal protease, tyrosine production, solid state- fermentation, plate assay, characterization, protease peptidyl aminopeptidase.

Resumo

A pesquisa atual foi projetada para alcançar o potencial de produção de protease extracelular em diferentes cepas de *Sordaria fimicola* que foram previamente obtidas do Dr. Lamb (Imperial College, Londres) de North Facing Slope e South Facing Slope de Evolution Canyon. Após a triagem inicial e secundária, duas cepas hiperprodutoras S2 e N6 foram selecionadas para fermentação submersa e condições culturais, incluindo temperatura, pH, período de incubação, tamanho do inóculo, concentração de substrato, e diferentes fontes de carbono e nitrogênio foram otimizadas para produção de enzima. A cepa S2 apresentou produção máxima de protease de 3,291 U/mL após 14 dias de incubação a 30 °C com pH 7, concentração de substrato de 1% e inóculo de 1 mL, enquanto a cepa N6 apresentou produção máxima de protease de 1,929 U/mL em condições otimizadas de fermentação. Outro objetivo da presente pesquisa foi sustentar a biodiversidade da genética e modificações pós-tradicionais (PTMs) da protease DPAP (peptidil-aminopeptidase) em *Sordaria fimicola*. Cinco sítios polimórficos foram observados na sequência de aminoácidos de cepas de *S. fimicola* com referência a *Neurospora crassa*. A previsão de PTMs a partir de ferramentas de bioinformática previu 38 locais de fosforilação em resíduos de serina para protease peptidil-aminopeptidase na cepa S1 de *S. fimicola*, enquanto 45 locais de fosforilação em serina na cepa N7 e 47 modificações de fosforilação de serina foram previstas em *N. crassa*. A pesquisa atual deu uma ideia de que a mudança na composição genética afetou os PTMs que, em última análise, afetaram a produção da enzima protease em diferentes cepas do mesmo organismo (*S. fimicola*). A produção e os dados moleculares da pesquisa revelaram que o estresse ambiental tem fortes efeitos sobre genes específicos por meio de mutações que podem causar diversidade genética. *S. fimicola* é um fungo não patogênico e tem um ciclo de vida curto. Esse fungo pode ser escolhido para produzir enzima protease em escala comercial.

Palavras-chave: produção e otimização de enzimas, protease fúngica, produção de tirosina, fermentação em estado sólido, ensaio em placa, caracterização, protease peptidil- aminopeptidase.

*e-mail: uzbifauzbifa@gmail.com
Received: August 25, 2021 – Accepted: November 11, 2021

1. Introduction

Bacteria and fungi are the good producers of industrial enzymes because of their biochemical and physiological characteristics, suitable culturing conditions and fast life span (Dias et al., 2008). The enzymes of microorganisms are always attracted by the modern era researchers due to their magnificent task in biological laboratories and industries. From a long time period, microorganisms are being used to fulfill the need of enzyme production at industrial level. Major industrial fungal species include ascomycetes and there is a big contribution of fungi for the manufacturing of enzymes for commercial use because the main focus of enzyme industry is to follow the most cost-effective methods to meet COGS (cost of goods sold) targets. Therefore when biological machineries are used for this purpose then it is important that it is cost efficient to use as raw material, easy to culture and produce high concentration enzymes (Arnau et al., 2020) and fungi are the best choice due to their utilization at broad spectrum in biotechnology industry for enzyme production as they are available excessively in nature with short generation time and easy gene handling as compare to higher organisms (Benmrad et al., 2019; Lange et al., 2020). Most of the fungi used in biotech-industry belong to ascomycetes which are in use from a long time for the manufacture of enzymes. However, the accessibility of genome sequences enlightened much more potentiality of ascomycetes in this industry and consequently, advanced omics-based research is under process (de Vries et al., 2020).

Proteases are combination of different enzymes which have commercial value due to their vital physiological applications. Due to large variety of proteases, in contrast to their particular actions, they have gained attention globally for their physiological and biotechnological utilization. Proteases are produced in bulk quantity contributing 66% of all enzymes employed on commercial scale (Hofmann et al., 2002). Proteases are produced by plants and animals, but scientific research always focused on the proteases production from microbes (Peng et al., 2003). The proteases produced by microbes are utilized in different industries like leather, detergent, brewing, food, waste management, photographic, chemical industries, silk degumming and in medical fields (Kalisz, 1988; Razzaq et al., 2019). The proteases produced from fungi have advantages over proteases produced by bacterium due to ease of hyphae isolation by filtration. The large number of industrial enzymes is available from fungi. Fungi like *Aspergillus oryzae* (Santhose et al., 2011; Vishwanatha et al., 2010), *Thermomyces lanuginosus* (Jensen et al., 2002), and *Mucor mucedo* (Santhose et al., 2011) are well known to produce protease. And most commonly used fungi for commercial production of chemicals and enzymes are filamentous in nature (Ma and Lee, 2008).

Fungi have ability to survive in changing environmental conditions and ecological scheme is linked with their enzyme secretion habit for obtaining nutrients (Muszewska et al., 2017) as variation in fungal enzyme production may be the correspondence of change in functions for environmental adaptations for endurance (Hu and Leger, 2004). By using molecular techniques scientists are trying to explore beneficial bio-molecules from fungi and their optimized conditions for industrial manufacturing and omics tools are very facilitating in recent researches because of their capability to define functional genomics, comparison of same genes between organisms, gene expression etc. (Bunnik and Le Roch, 2013; McCluskey et al., 2010). Results obtained from omics studies have given a great insight about the interactions between environmental factors, genetic diversity, and variation in concentration of metabolites *etc.* (Téllez-Téllez and Diaz-Godinez, 2019). In current study *Sordaria fimicola* strains collected from north and south facing slopes of evolution canyon are used for protease production under optimized conditions to check the variation of protease enzyme activity, biomass production and diversity in their genes and as well as their post translational modifications by using bioinformatics tools.

2. Research Design and Methodology

2.1. Collection of samples

Samples of *S. fimicola* were provided by Molecular Genetics Research Lab, Botany department, Punjab University, Lahore which were previously obtained from Dr. Lamb (Imperial College, London) collected from North Facing Slope and South Facing Slope of Evolution Canyon.

2.2. Screening of protease by plate assay method and comparative production of protease under submerged fermentation conditions

The fungal strains were screened for proteases by Hankin and Anagnostakis method in which gelatin was used as a source of protein in growth medium (Hankin and Anagnostakis, 1975).

2.3. Optimization of fermentation conditions for the production of protease

The strains of *S. fimicola* were cultured in 500 mL conical flask containing medium of different pH ranges from 2.0 to 9.0. The flasks were kept at 20 °C for 21 days. In case to find out the effective temperature for the production of protease by different strains of *S. fimicola*, fermentation was carried out at different temperatures ranging 10 °C to 60 °C. Similarly, substrate concentration by taking different concentrations of fungal spores in dis. H_2O (0.5 mL, 1 mL, 1.5 mL, 2.0 mL, 2.5 mL, 3.0 mL) and inoculums size by taking different sizes of disc of fungus (0.5 cm, 0.8 cm, 1.0 cm, 1.2 cm and 1.4 cm) were also optimized. Then the effects of carbon sources were checked by taking 3g glucose, 3g maltose and 3g of lactose separately in flasks containing 1.5g PDB, 1.5g Casein in 150 mL of distilled water at 30 °C and pH 6. Similarly the effects for Nitrogen sources have been checked by taking 3g Peptone, 3g beef extract and 3g of ammonium sulphate separately.

2.4. Assay for the activity of protease

Proteases activity in the crude enzyme extract was determined according to the method of Cupp- Enyard

(Cupp-Enyard 2008) by using Casein as a substrate and one protease unit was defined as the amount of enzyme that releases 1 µM of tyrosine per minute at pH 7.5 at 37 °C (Mohapatra et al., 2003). All the experiments were done in triplicates and mean value was calculated.

2.5. Extraction of proteases from fermentation media and determination of enzyme activity and specificity

The filtrates of fermentation media were then centrifuged at 5000 rpm for 20 minutes. And the supernatant obtained was filtered through whatman filter paper No. 1 so as to obtain a cell free supernatant which was used as a source of crude enzyme for estimation of protease (Kheng and Omar, 2005).

Total activity (total U) = µmole/ mL x filtrate volume. The specific activity was expressed in total U/total mg protein of the extracellular extract.

2.6. Determination of biomass

After submerged fermentation the biomass was collected by filtering the cultural medium through pre-weighed Whatman filter paper no. 1. The extract was oven dried at 80 °C and reweighed. The difference in the weight was the biomass of fungus.

2.7. DNA Extraction, amplification and sequencing

DNA extraction of *S. fimicola* strains was done by adopting the protocol of Saghai-Maroof et al. (1984). The concentration of DNA was estimated by measuring the absorbance at 260nm. The quality of genomic DNA was estimated by the ratio OD260/OD280. Primers were designed through Primer 3 software. By using forward primer sequence ATGCCATCCACGTACTCGGACG and reverse primer sequence TCACTTCATATTGGCCAACAAAT amplification was carried out under the following PCR reaction conditions: one round of amplification consisting of denaturation at 95 °C for 15 min followed by 30 cycles of denaturation at 95 °C for 20 sec, annealing at 50 °C for 40 sec and extension at 72 °C for 1 min, with a final extension step of 72 °C for 5 min. The PCR products were confirmed by gel electrophoreses on 0.8% agarose gel. The bands were eluted and sequenced.

2.8. Post-translation modifications prediction tools

PTMs were investigated by using various bioinformatics tools including YinOYang 1.2 Server; (DTU Health Tech, 2021a). NetPhos 3.1 Server (DTU Health Tech, 2021b) and NetNES 1.1 Server (https://services.healthtech.dtu.dk) to predict glycosylation, phosphorylation sites and nuclear export signals (NES) respectively. The sequences of amino acids of amplified genes were obtained from an online tool "EMBOSS Transeq" (EMBOSS Transeq, 2021) while the sequence of amino acids of the reference strain *N. crassa* was retrieved from Uniprot.

2.9. The homology modelling and model validation

PyMol is a reliable 3D structure prediction tool, which was used to build 3D models of protease dipeptidyl aminopeptidase with 100% confidence prediction.

3. Results

3.1. Biochemical analysis

3.1.1. Comparative initial and secondary protease screening among fungal strains

In initial screening maximum zones of hydrolysis were observed in N6 and S2 strains while N5 and N7 strains showed minimum zones as shown in Graph 1. Secondary screening for protease production under submerged fermentation among fungal strains also showed maximum biomass production and enzyme activity in S2 and N6 strains (Graph 2).

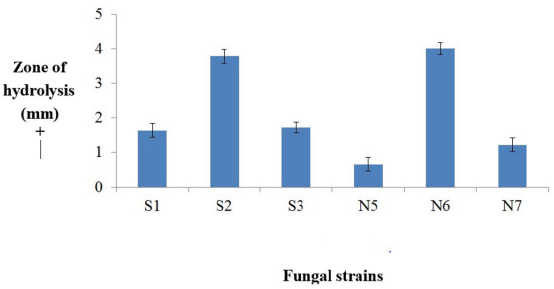
3.2. The optimization of cultural conditions for the production of protease

3.2.1. The effect of temperature

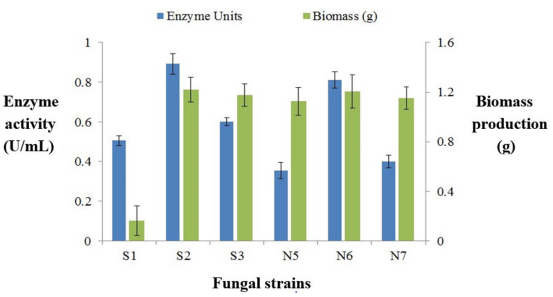
Different ranges of temperatures also affect the enzyme activity and biomass production. In our work, enzyme activity showed a trend of 30 °C >20 °C >40 °C respectively (Graph 3.1). Same trend was observed in case of biomass production as well in both S2 and N6 strains (Graph 3.2).

3.2.2. The effect of pH

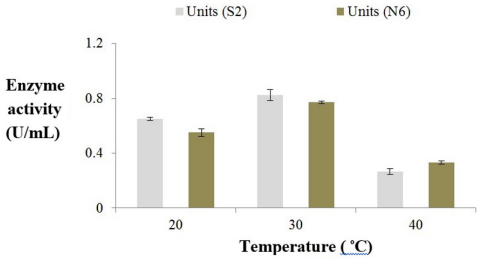
pH change affect enzyme activity as at 7 pH enzyme activity was maximum and at pH 5 it was minimum (Graph 4.1) but biomass production was highest at pH 7 and lowest at pH 6 (Graph 4.2).



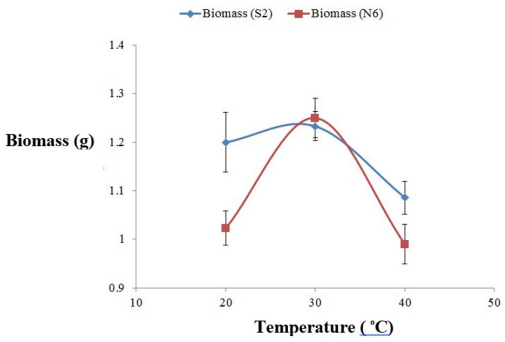
Graph 1. Comparison of initial screening for protease production among fungal strains.



Graph 2. Comparison of secondary screening for protease production under submerged fermentation among fungal strains.

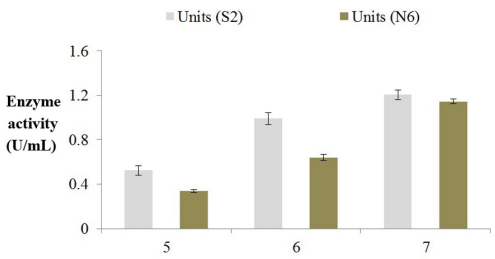


Effect of temperature on enzyme activity

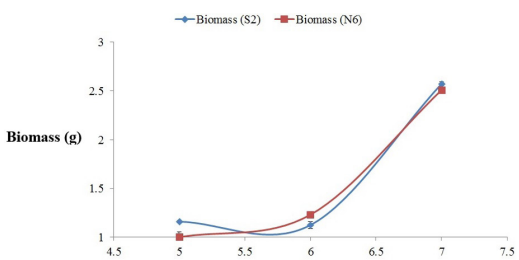


Effect of temperature on biomass production

Graph 3. 3.1 Comparison of Protease production by S.fimicola (S2 and N6) a±t various temperatures under submerged fermentation. These experiments were done in triplicates and values are with ± SD. 3.2 Effect of various temperatures on S.fimicola strains (S2 an±d N6) biomass production during submerged fermentation. All the experiments were done in triplicates and values with ± SD.



Effect of different pH on enzyme activity



Effect of different pH on biomass production

Graph 4. 4.1 Effect of various temperatures on S.fimicola strains (S2 an±d N6) biomass production during submerged fermentation. All the experiments were done in triplicates and values with ± SD. 4.2 Effect of different pH on biomass productions by S.fimicola strains (S2 and N6) using submerged fermentation. These experiments were done in triplicates and values with ± SD.

3.2.3. The effect of incubation time

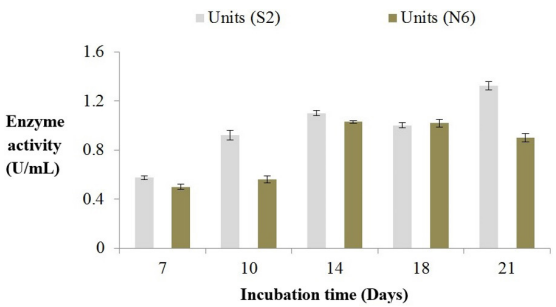
Maximum to minimum values of enzyme activity were observed on 21 days >14 days >18 days > 10 days > 7 days in S2 strain and on 14 days > 18 days >21 days > 10days >7 days incubation time in N6 strain respectively (Graph 5.1). Protease biomass production values were highest to lowest on 14 days > 21 days >18 days > 10 days >7 days in S2 strain and on 14 days >18 days > 21 days >10 days > 7 days in N6 strain respectively (Graph 5.2).

3.2.4. The effect of inoculum volume

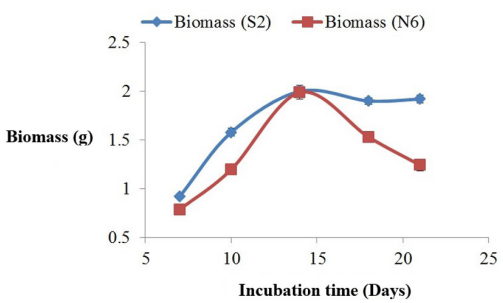
Enzyme activity was highest with 1.5 mL inoculum volume in N6 and 1 mL inoculum in S2 strains. Least enzymatic activity was with 2.5 mL and 3 mL inoculum in N6 and S2 respectively (Graph 6.1). Biomass production was maximum with 1.5 mL and 0.5 mL inoculum in N6 and S2 respectively and both strains showed minimum biomass production with 3 mL inoculum volume (Graph 6.2).

3.2.5. The effect of concentration of substrate

As the concentration of substrate (Casein) was increased to 4%, the results showed minimum enzyme activity and as the concentration of substrate was decreased to 1% its enzyme activity was maximum in both strains N6 and S2 (Graph 7.1). In case of biomass production, 1% concentration was found to be most effective for N6 and 2% concentration was found to be most effective for



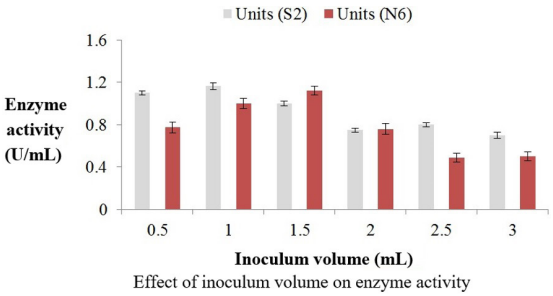
Effect of incubation time on enzyme activity



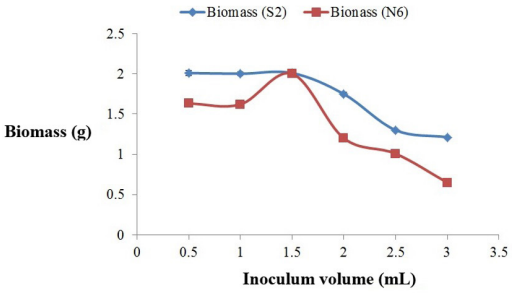
Effect of incubation time on biomass production

Graph 5. 5.1 Determination of effect of various incubation time on protease activity by hyper-producer S.fimicola strains (S2 and N6) under submerged fermentation. These experiments were done in triplicates and values with ± SD. 5.2 Effect of various incubation periods on S.fimicola strains (S2 and N6) biomass production. These experiments were done in triplicates and values with ± SD.

S2 strain while least effectiveness was observed at 0.5% concentration in both strains (Graph 7.2).

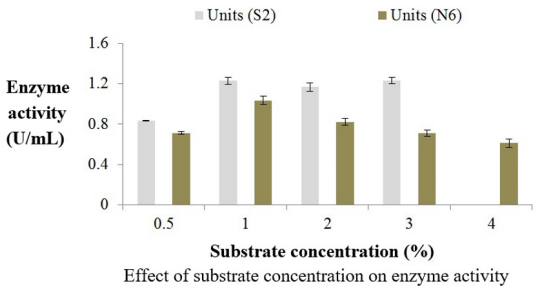


Effect of inoculum volume on enzyme activity

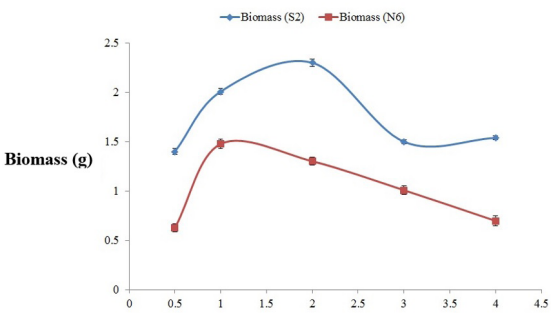


Effect of inoculum volume on biomass production

Graph 6. 6.1 S.fimicola strains showing different behavior of protease production due to different inoculum concentrations. These experiments were done in triplicates and values with ± SD. 6.2 S.fimicola strains showing different behavioue of biomass production on different inoculum concentrations (mL) with ± SD.



Effect of substrate concentration on enzyme activity



Effect of substrate concentration on biomass production

Graph 7. 7.1 Substrate concentrations'effect on the production of protease by S2 and N6 respectively. 7.2 S.fimicola strains (S2 and N6) showing different biomass production on various substrate concentrations with ± SD.

3.2.6. The effect of carbon sources

According to the results shown in Graph 8.1, glucose is found to be the most effective carbon source among all other sources examined for enzyme and biomass production in both S2 and N6 strains (Graph 8.2).

3.2.7. The effect of nitrogen source

Graph 9.1 and 9.2 indicate that S2 strain showed maximum enzyme activity and biomass production with peptone as nitrogen source in growth media and N6 strain showed maximum enzyme activity and biomass production with beef extract as nitrogen source in growth media.

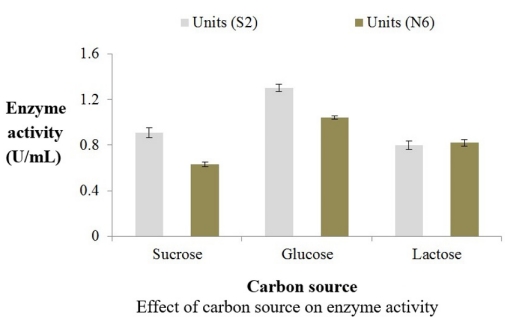
3.3. Comparative effect of optimized conditions on the protease enzyme activity in S. fimicola

Fungal strains with optimized conditions showed very high enzyme activity in S2 strain than in N6 strain. Total protein quantity was also high in S2 as compared to N6 and protease enzyme specificity was almost equal in both strains (Graph 10).

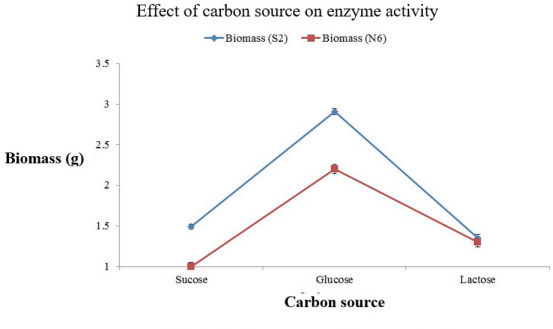
3.4. Molecular analysis

The dipeptidyl aminopeptidases are proteases of cysteine that cleave dipeptides from the N-terminus of the substrates of protein. The dipeptidyl aminopeptidase A is present in golgi apparatus, is responsible for processing of a factor in *Saccharomyces cerevisiae* and also helps in protein maturation (Anna-Arriola and Herskowitz 1994).

The genomic DNA of all strains of *S.fimicola* was used to amplify the protease-dipeptidyl-aminopeptidase

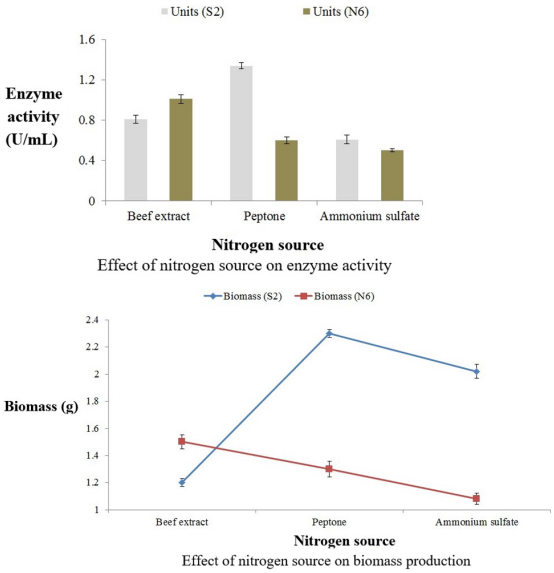


Effect of carbon source on enzyme activity

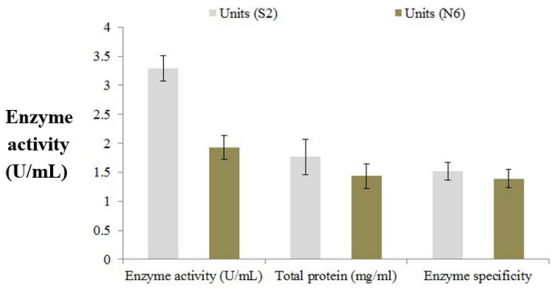


Effect of carbon source on biomass production

Graph 8. 8.1 Different enzymes activities of two fungal strains on three carbon sources under submerged fermentation. These experiments were done in triplicates and values with ± SD. 8.2 Effect of different Carbon sources on the biomass of S.fimicola strains. These experiments were done in triplicates and values with ± SD.



Graph 9. 9.1 Effect of various Nitrogen sources on enzyme activities of two fumgal strains. These experiments were done in triplicates and values with ± SD. 9.2 S.fimicola strains (S2 and N6) showing different behaviour for biomass production in three different nitrogen sources. These experiments were done in triplicates and values with ± SD.



Graph 10. Comparison of enzyme activities of both fungal strains at optimized conditions.

```
S3  ACGAGTATCGTCTTCGATCGCATACAAGAGCGTCTCGATACCAAGGAGTTTCCGGCTCGC  180
S1  ACGAGTATCGTCTTCGATCGCATACATGAGCGTCTCGATACCAAGGAGTTTCCGGCTCGC  180
S2  ACGAGTATCGTCTTCGATCGCATACATGAGCGTCTCGATACCAAGGAGTTTCCGGCTCGC  180
NC  ACGAGTATCGTCTTCGATCGCATACAAGAGCGTCTCGATACCAAGGAGTTTCCGGCTCGC  180
N7  ACGAGTATCGTCTTCGATCGCATACATGAGCGTCTCGATACCAAGGAGTTTCCGGCTCGC  180
N5  ACGAGTATCGTCTTCGATCGCATACAAGAGCGTCTCGATACCAAGGAGTTTCCGGCTCGC  180
N6  ACGAGTATCGTCTTCGATCGCATACATGAGCGTCTCGATACCAAGGAGTTTCCGGCTCGC  180
    ****************************** ******* **************************************************

S3  ACTGGGCCCTTCTTGGGTAATGGGAGCCCCTCTTCCCGTTCAAACCAGCGCTCATCCGCC  300
S1  ACTGGGCCCTTCTTGGGTAATGGGAGCCCCTCTTCCCGTTCAAACCAGCGCTCATCCGCC  300
S2  ACTGGGCCCTTCTTGGGTAATGGGAGCCCCTCTTCCCGTTCAAACCAGCGCTCATCCGCC  300
NC  ACTGGGCCCTTCTTGGGTAATGCGAGCCCCTCTTCCCGTTCAAACCAGCGCTCATCCGCC  300
N7  ACTGGGCCCTTCTTGGGTAATGGGAGCCCCTCTTCCCGTTCAAACCAGCGCTCATCCGCC  300
N5  ACTGGGCCCTTCTTGGGTAATGCGAGCCCCTCTTCCCGTTCAAACCAGCGCTCATCCGCC  300
N6  ACTGGGCCCTTCTTGGGTAATGGGAGCCCCTCTTCCCGTTCAAACCAGCGCTCATCCGCC  300
    ****************************** ************************************************

S3  CTAGACCAAGTCTTAAACAATGTATGGCGTGCCAAGAGCCATTCCATCAGCTGGATCGCT  540
S1  CTAGACCAAGTCTTAAACAATGTATGGCGTGCCAAGAGCCATTCCATCAGCTGGATCGCT  540
S2  CTAGACCAAGTCTTAAACAATGTATGGCGTGCCAAGAGCCATTCCATCAGCTGGATCGCT  540
NC  CTAGACCAAGTCTTAAACAATGAATGGCGTGCCAAGAGCCATTCCATCAGCTGGATCGCT  540
N7  CTAGACCAAGTCTTAAACAATGAATGGCGTGCCAAGAGCCATTCCATCAGCTGGATCGCT  540
N5  CTAGACCAAGTCTTAAACAATGAATGGCGTGCCAAGAGCCATTCCATCAGCTGGATCGCT  540
N6  CTAGACCAAGTCTTAAACAATGAATGGCGTGCCAAGAGCCATTCCATCAGCTGGATCGCT  540
    ************************ ****** **************************************************

S3  GGTGTAAACGGAGAAGATGGGCTTCTTCTAGAGAAGGAGGGCGCAAATAAAGACTACCTT  600
S1  GGTGTAAACGGAGAAGATGGGCTTCTTCTAGAGAAGGAGGGCGCAAATAAAGACTACCTT  600
S2  GGTGTAAACGGAGAAGATGGGCTTCTTCTAGAGAAGGAGGGCGCAAATAAAGACTACCTT  600
NC  GGTGTAAACGGAGAAGATGGCCTTCTTCTAGAGAAGGAGGGCGCAAATAAAGACTACCTT  600
N7  GGTGTAAACGGAGAAGATGGCCTTCTTCTAGAGAAGGAGGGCGCAAATAAAGACTACCTT  600
N5  GGTGTAAACGGAGAAGATGGCCTTCTTCTAGAGAAGGAGGGCGCAAATAAAGACTACCTT  600
N6  GGTGTAAACGGAGAAGATGGCCTTCTTCTAGAGAAGGAGGGCGCAAATAAAGACTACCTT  600
    ************************** ************************************************

S3  CCCTACGATGCCGATGCTCGTCTCCAGCTGGCGTCATGGAGGCCCACCAGTGATGCCATT  900
S1  CCCTACGATGCCGATGCTCGTCTCCAGCTGGCGTCATGGAGGCCCACCAGTGATGCCATT  900
S2  CCCTACGATGCCGATGCTCGTCTCCAGCTGGCGTCATGGAGGCCCACCAGTGATGCCATT  900
NC  CCCTACGATGCCGATGCTCGTCTCCAGCTGGCGTCATGGAGCCCCACCAGTGATGCCATT  900
N7  CCCTACGATGCCGATGCTCGTCTCCAGCTGGCGTCATGGAGGCCCACCAGTGATGCCATT  900
```

Figure 1. Multiple sequence alignment of nucleotide sequence of six strains of *Sordaria fimicola* with reference sequence of *Neurospora crassa.* The gaps or spaces are showing polymorphic sites and symbols (*) are showing similar or non-polymorphic sites.

gene, this gene (protease-dipeptidyl-aminopeptidase gene) was chosen from *N. crassa* (a reference organism) from NCBI, Gene name is NCU02515 and Gene ID is 3881644, its description is dipeptidyl-aminopeptidase (Neurospora crassaOR74A), Location Chromosome I, NC-026501.1 2584409..2587786, and after PCR product size of 1473 bp was obtained. Polymorphism study was carried out by aligning the nucleotide sequences of different *S. fimicola* strains with *N. crassa* (a reference organism) in Clustal Omega software. The numbers of polymorphic sites in all strains of *S. fimicola* with respect to *N. crassa* are 18 (Figure 1). After sequencing, the sequences were subjected to blast tool at NCBI to check homologous sequences to those found in *S. fimicola*. BLAST used the *S. fimicola* sequence as query sequence to find out the homologous region in *N. crassa*. The multiple sequence alignment of the amino acid sequence of *S. fimicola* strains with *N. crassa* (a reference species) was obtained. The alignment of the amino acid sequences of six strains of *S. fimicola* with the reference sequence of *N. crassa* showed five polymorphic sites and three sites were found to be highly conserved among the species of strongly similar properties. The gaps are showing polymorphic sites and symbol (:) is showing the conservation among the species of strongly similar properties. The asterisk (*) shows that stop codons are present here (Figure 2).

3.5. PTMs analysis

All possible glycosylation and phosphorylation sites of *N. crassa* and *S. fimicola* are given in Table 1. The results of glycosylation are predicted by YinOYang server. We detected many conserved sites by comparing the glycosylation sites of both strains. These sites were defined on Serine (S-98, S -117, S–133, S–140, S–141, S–211, S–212, S–561, S-686, S-690)

```
NC_1   GTDGDDNDSLKDELNNDDLETGPFLGNASPSSRSNQRSSADGQRMDRSLRRWLFIVSGAL   120
N7_1   GTDGDDNDSLKDELNNDDLETGPFLGNGSPSSRSNQRSSADGQRMDRSLRRWLFIVSGAL   120
S1_1   GTDGDDNDSLKDELNNDDLETGPFLGNGSPSSRSNQRSSADGQRMDRSLRRWLFIVSGAL   120
S2_1   GTDGDDNDSLKDELNNDDLETGPFLGNGSPSSRSNQRSSADGQRMDRSLRRWLFIVSGAL   120
N5_1   GTDGDDNDSLKDELNNDDLETGPFLGNASPSSRSNQRSSADGQRMDRSLRRWLFIVSGAL   120
N6_1   GTDGDDNDSLKDELNNDDLETGPFLGNGSPSSRSNQRSSADGQRMDRSLRRWLFIVSGAL   120
       ************************************ * : * *** ************************************

NC_1   VYTRDNNMFLRKLDSDKIVQITRDGSADVFNGVPDWVYEEEVLASGVATWWSEDGNYVAF   360
N7_1   VYTRDYNMFLRKLDSDKIVQITSDGSADVFNGVPDWVYEEEVLASGVATWWSEDGNYVAF   360
S1_1   VYTRDYNMFLRKLDSDKIVQITSDGSADVFNGVPDWVYEEEVLASGVATWWSEDGNYVAF   360
S2_1   VYTRDYNMFLRKLDSDKIVQITSDGSADVFNGVPDWVYEEEVLASGVATWWSEDGNYVAF   360
N5_1   VYTRDYNMFLRKLDSDKIVQITSDGSADVFNGVPDWVYEEEVLASGVATWWSEDGNYVAF   360
N6_1   VYTRDYNMFLRKLDSDKIVQITSDGSADVFNGVPDWVYEEEVLASGVATWWSEDGNYVAF   360
       ****** ***************** *****  ************************************************

NC_1   DAGQTFKYGMAVAPVTDWRFYDSIYTERYMRTPQTNPEGYESAAVTNVTALSQNVRFLLM   840
N7_1   DAGQTFKYGMAVAPVTDWRFYDSIYTDRYMRTPQTNPEGYESAAVTNVTALSQNVRFLLM   840
S1_1   DAGQTFKYGMAVAPVTDWRFYDSIYTDRYMRTPQTNPEGYESAAVTNVTALSQNVRFLLM   840
S2_1   DAGQTFKYGMAVAPVTDWRFYDCIYTDRYMRTPQTNPEGYESAAVTNVTALSQNVRFLLM   840
N5_1   DAGQTFKYGMAVAPVTDWRFYDSIYTDRYMRTPQTNPEGYESAAVTNVTALSQNVRFLLM   840
N6_1   DAGQTFKYGMAVAPVTDWRFYDSIYTDRYMRTPQTNPEGYESAAVTNVTALSQNVRFLLM   840
       ************************.****.***.:***  ********************************************

NC_1   HGVADDNVHMQNSLTLLDALDQRSVENYDVQVFPDSDHGIYFHNANRIVFDKLTNWLVNA   900
N7_1   HGVADDNVHMQNSLTLLDALVQRSVENYDVQVFPDSDHGIYFHNANRIVFDKLTNWLVNA   900
S1_1   HGVADDNVHMQNSLTLLDALDQRSVENYDVQVFPDSDHGIYFHNANRIVFDKLTNWLVNA   900
S2_1   HGVADDNVHMQNSLTLLDALDQRSVENYDVQVFPDSDHGIYFHNANRIVFDKLTNWLVNA   900
N5_1   HGVADDNVHMQNSLTLLDALDQRSVENYDVQVFPDSDHGIYFHNANRIVFDKLTNWLVNA   900
N6_1   HGVADDNVHMQNSLTLLDALDQRSVENYDVQVFPDSDHGIYFHNANRIVFDKLTNWLVNA   900
       ********************* ******** : * ************************************************

NC_1   FNGEWLKIANAQPNGMKRRALPTA*----------                             924
N7_1   FNGEWLKIANAQPNGMKRRALPTA*----------                             924
S1_1   FNGEWLKIANAQPNGMKRRALPTA*----------                             924
S2_1   FNGEWLKIANAQPNGMKRRALPTA*SMPSTYSDDNTLRSGLDRFRDHSPSQHRRSMSQET   959
N5_1   FNGEWLKIANAQPNGMKRRALPTA*----------                             924
N6_1   FNGEWLKIANAQPNGMKRRALPTA*----------                             924
       *************************
```

Figure 2. Multiple sequence alignment of amino acid sequence of six strains of *Sordaria fimicola* with reference sequence of *Neurospora crassa.* The gaps are showing polymorphic sites and symbol (:) is showing the conservation among the species of strongly similar properties.

and on Threonine (T- 4, T-152, T-239, T-585, T-796, T-815, T-923). Some non-conserved regions were predicted in *N. crassa* on Serine and Threonine (S-91, T-297). All strains of *S. fimicola* also had non-conserved regions on Serine and Threonine which are (S-173, T-322).

We found many sites conserved by comparing the phosphorylation sites of both strains. The conserved regions were defined on serine between *N. Crassa* and *S. fimicola*'s strain S2, S3 (S-23,S-91, S-94, S-96, S-155, S-175, S-177). The conserved regions on Threonine (T-589) have been detected between *N. crassa* and *S. fimicola* strain S1. The sites (S-7, S-14, T-11, T-63, T-212, T-274, T-924) of *S. fimicola* are different from *N. crassa* after PTMs due to the genetic variation. The sites (S-8, T- 12, T-239, Y-12, Y-56, and Y-786) of N. crassa are different from *S. fimicola* due to genetic variations. Nuclear export signals on residue 250-L and 275-M in *N. crassa* and *S. fimicola* have been predicted in Figure 3 and 4.

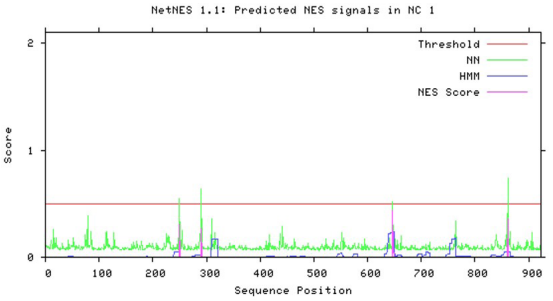


Figure 3. Graphical representation of leucine rich nuclear export signals (NES) for of *N. crassa*. Green peaks are showing NN signals, blue peaks are showing HMM signals, purple peaks are showing NES signals and red line is showing threshold level, which is 0.5 and above.

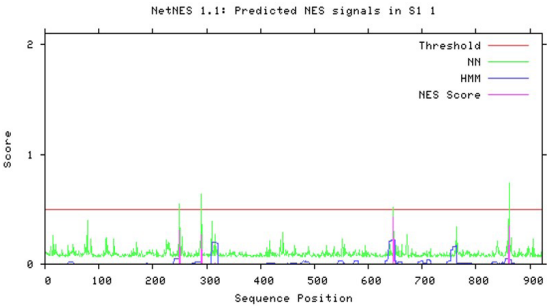


Figure 4. Graphical representation of leucine rich nuclear export signals (NES) for of *S. fimicola*. Green peaks are showing NN signals, blue peaks are showing HMM signals, purple peaks are showing NES signals and red line is showing threshold level, which is 0.5 and above.

3.6. Molecular modelling and structure validation

3D models of protease dipeptidyl aminopeptidase were visualized in PyMOL. Sticks represents coil structure, arrows represent β - sheet and sticks and cartoon coils indicates α - helix. (a). *N. crassa* with dimensions (Å):X:89.158 Y:70.858 Z:86.334. (b) *S. fimicola* S1 having dimensions (Å):X:89.158 Y:70.085 Z:86.043. (c). *S. fimicola* N5 showing dimensions (Å): X: 89.158 Y: 69.975 Z:86.334 in Figure 5.

4. Discussion

The current research investigated the genes involved in production of proteases from *S. fimicola*. This study helped to search out the posttranslational modifications and their impact on the multifunctionality of proteases. The second purpose of this study was to find out whether six strains of *S. fimicola* obtained from different environmental stresses possess tendencies of generating genetic variations or not. In the current research, used *S. fimicola* strains were collected from two slopes of different fauna and flora. North-facing slope (NFS) has moister (mesic) and lush green climate whereas South facing slope (SFS) has harsh and dry (xeric) climate (Ishfaq et al., 2017).

Table 1. Table is showing predicted O-glycosylation and phosphorylation sites at serine, threonine and tyrosine residues for protease dipeptidly aminopeptides of *N.cressa* and *S. fimicola* (Server Netphos 3.1).

Organism	Residue	Glycosylation	Phosphorylation
N.cressa	Serine	91*,92,95*,98,113*,117,140,	5,8*,15,24,26,31,33,44,91,93,94,96,100
	(S)	141,211,212,561,585*,689*,	101,102,119,135,136,000,000,000,000
		690	177,179,213,214,218,000,000,000,000
		Total= 14	328,383,426,430,459,000,000,000,000
			584,600,692,700,705,000,000
			Total= 47
	Threonine	4,152,239,297*,585,796,815,	2,36,38,41,56,64,154,162,222,239*,275
	(T)	923	368,455,473,563,564,589*,694,717,723
		Total= 8	776,787,798,815
			Total= 24
	Tyrosine	--	12*,38,41,56*,64,368,473,563,564,589
	(Y)		598,622,623,694,723,776,786*,814
			Total= 18
S.fimicola SI	Serine	92,98,117,133,140,141,173*,	5,7*,14*,23*,25,30,32,37,40,43,55,92,100
	(S)	211,212,297*,561,686*,690	142,156,176,178,212,000,000,000,000
		Total= 13	293,298,316,324,382,000,000,000,000
			567,597,599,691,704,000,000
			Total= 38
	Threonine	4,152,239,322*,585,796,815	11*,63*,153,161,212*,238,274*,454,472,
	(T)	923	563,583,588,589*,622,693,716,722,775
		Total= 8	786,797,816,924*
			Total=22
	Tyrosine	--	200,297,358,393,511,000,000,000,000
	(Y)		629,665,712,757,773,000,000,000
			Total= 17
S2,S3	Serine	92,98,117,133,140,141,173*,	6,13,22,24,29,31,36,39,42,9*1,92,94*,96*
	(S)	211,212,297*,561,686*,690	98,99,108,117,133,134,141,155*,173,
		Total= 13	175*,177*,211,212,216,261,292,297,323
			381,428,457,548,561,000,000,000,000
			690,703,832,853,864
			Total= 45
	Threonine	4,152,239,322*,585,796,818,	4,10,34,54,62,152,160,220,273,296
	(T)	922	366,453,471,582,587,000,000,000,000
		Total= 8	785,796,815,923
			Total= 23
	Tyrosine	--	199,357,392,510,541,000,000,000,000
	(Y)		664,711,756,772,805,000,000
			Total= 16
N5,N6 & N7	Serine	92,98,117,133,140,141,173*,	6,13,22,24,29,31,36,39,42,91,92,94,96
	(S)	212,297*,561,686*,690	98,99,108,117,133,134,141,155,173,175
		Total= 12	177,211,212,216,261,000,000,000,000
			428,457,548,561,566,000,000,000,000,000

The symbols (*) are showing similar or non-polymorphic sites.

Table 1. Continued...

Organism	Residue	Glycosylation	Phosphorylation
			832,853,864
			Total= 45
	Threonine	4,152,239,322*,584,585,	4,10,34,54,62,152,160,220,273,296,366,
	(T)	796,814*,815*,922,923*	453,471,582,587,621,000,000,000,000,000
		Total= 11	815,923
			Total: 23
	Tyrosine	--	199,357,392,510,541,562,592,593,628,664,
	(Y)		711,756,772,805,809,000
			Total=16

The symbols (*) are showing similar or non-polymorphic sites.

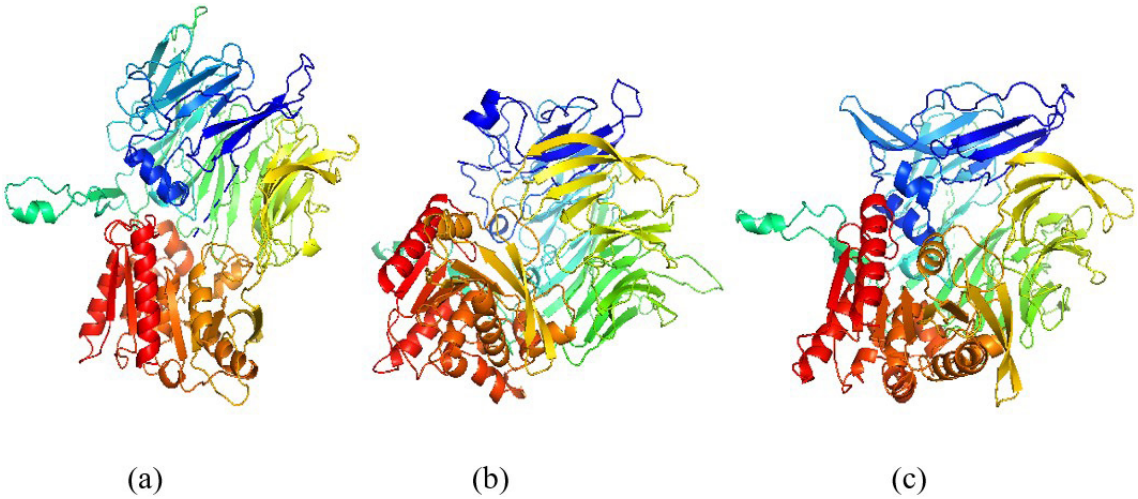


Figure 5. 3D models of protein dipeptidyl aminopeptidase visualized in PyMOL.Sticks represents coil structure, arrows represents β - sheet and sticks and cartoon coils indicates α - helix. (a) *N.crassa* with dimensions (Å):**X**:89.158 **Y**:70.858 **Z**:86.334; (b) *S.fimicola*S_1 having dimensions (Å):**X**:89.158 **Y**:70.085 **Z**:86.043; (c) *S.fimicola*N_5showing dimensions (Å): **X**:89.158 **Y**:69.975 **Z**:86.334.

The screening of *S. fmicola* of its protease enzyme activity was carried out with the application of Plate assay method by the substrate (casein) hydrolysis in the medium. The activities of enzyme were studied by the presence of clear zones around the *S. fmicola* colonies after an incubation period. *S. fimicola* S2 and *S. fimicola* N6 out of the total six strains presented largest zone around the colony of fungus, so they were selected for next work. *Aspergillus*, *Penicillium*, *Rhizopus*, and *Rhizomucor* produce proteases in large quantities (Krishna et al., 2009; Sindhu et al., 2009) and are efficient over a wide range of pH (Rao et al., 1998).

Incubation temperature has a very significant effect in enzyme production. The minute change in temperature has large influence on the production of enzymes. The protease production on optimal temperature was checked by incubation of the growth medium at different temperatures, at ranges of 20 °C, 25 °C, 30 °C, 35 °C, 40 °C, and 45 °C. The production of protease was found to be highest at 30 °C. The results of different temperatures revealed that the optimum temperature for protease production in S2 (0.824 U/mL) and N6 (0.77 U/mL) was 30 °C (Graph 3).

At high temperature, fungal growth and enzyme production, both are very poor (Sabu et al., 2002). Kumar and his colleagues found that fungal proteases produced at less than 40 °C (Kumar et al., 2008). Morimura and his colleagues also described that fungus growth and proteases production are maximum at 50 °C. This study has exposed the reality that *S. fimicola* can be used for production of protease on commercial scale. The remarkable high enzyme activity at pH 7.0 suggested that *S. fimicola* is a potential producer of proteases which is very beneficial in various industries (Morimura et al., 1994).

pH has also very strong effect on the morphological changes in fungus and also in enzyme production. *S. fimicola* strains grew in media of different pH ranging from 4.0 to 9.0. Scientists of modern era have reported that the microbes produce acidic or alkaline proteases. The optimum pH

for protease activity was between 7 and 8.5, reported by (Nascimento and Martins, 2004; Sookkheo et al., 2000). The extreme low and high pH cause instability of enzymes (Abidi et al., 2008). The protease production was best observed initially at pH 6.0 (Karthic et al., 2014). In our research, the best enzyme production was detected at pH 7.0 in case of *S. fimicola* S2 (1.205 U/mL) and *S. fimicola* N6 (1.145 U/mL) (Graph 4.1).

In this research, the incubation period was checked for enzyme production from 7 to 21 days. S2 showed more enzyme activity 1.325 U/mL after 14 days as compared to N6 1.030 U/mL. Sathya et al. (2009) reported that Mucor circinelloids showed maximum protease production between 5 and 6 days. Abidi and his colleagues reported that the fungus *Botrytis cinerea* expressed a large extracellular proteases activity during first 6 days and obtained the maximum position after 9 days (Abidi et al., 2008).

Inoculum size in fungal fermentation is also very important. Five inoculum sizes (0.25 mL, 0.50 mL, 0.75 mL, 1.0 mL and 1.25 mL) were used but 1.0 mL inoculum size was found to be best for maximum protease production by *S. fimicola*. The enzyme activity was 1.166 U/mL which was highest at 1.0 mL inoculum size and minimum enzyme activity which was 0.703 U/mL, showed at 3.0 mL of inoculum size. The inoculums concentration reveals the strong effects on the protease production, depending upon the strain's properties of microorganisms. The maximum protease production (1.166 U/ mL) was expressed in 1.0 mL of initial inoculum size. The current research results are similar with the results of (Mohapatra, Bapuji and Sree 2003).

Protease activity was observed at different concentrations of substrate and it was checked that 1% casein act as the best substrate concentration for both the strains of *S. fimicola* S2 and N6 for the highest protease production. S2 expressed highest enzyme activity of 1.230 U/mL than that of N6 which showed 1.034 U/mL activity at 1% substrate concentration. Samarntarn and his colleagues reported that low concentration of casein produces less amounts of protease and at high concentration casein acts as a nitrogen metabolite repressor in several types of yeast (Samarntarn et al., 1999).

In current study, *S. fimicola* S2 (1.301 U/mL) and N6 (1.041 U/mL) showed best results on 3% glucose, whereas protease production was also good at 3% sucrose and lactose concentration. The sources of carbon also possessed good effects on the proteases production. The scientists reported that the better proteases production occurs if glucose used as a source of carbon (Ferrero et al., 1996; Mehrotra et al., 1999). The enzymes secretions are affected by nitrogen sources (Mehta et al., 2006; Vinogradova et al., 2003). Various nitrogen sources such as beef extract, peptone and $(NH4)_2SO_4$ had good effects on *S. fimicola* proteases synthesis. But in current research, peptone strongly increased proteases production in *S. fimicola*. The results of current studies showed that 3% peptone was best for protease production by *S. fimicola* S2 (1.340 U/mL) whereas 3% beef extract was best for protease production of *S. fimicola* N6 (1.0 U/mL). The production of protease without nitrogen source was low (0.4 U/mL) that revealed the requirement of sources of nitrogen for the growth of *S. fimicola* and also the production of protease.

In current molecular genetics study, protease dipeptidyl aminopeptidases gene was amplified in all strains of *S. fimicola*. The genomic sequence of *Sordaria* species and *N. crassa* are closely related to each other (Nowrousian et al., 2010). All possible glycosylation sites of *N. crassa* and *S. fimicola* are given in Table 1. The role of glycosylation of protein is very essential in various biological processes like protein folding, and cell adhesion (Lee et al., 2015). The changes in the glycosylation pattern may causes diseases such as neurodisorders, cancer and diabetes (Pinho and Reis 2015). The results of glycosylation are predicted by YinOYang Server. We detected many conserved sites by comparing the glycosylation sites of both strains. Some sites were defined on Serine (S-98, S-117, S–133, S–211, S–212, S–561, S-690) and Threonine (T-4, T-239, T-585, T-815, T-923) between *N. crassa* and *S. fimicola* strains. Bukhari et al. (2020) reported the glycosylation sites on threonine (T-173, T-279) residues in cytochrome C-1 (CyC-1) of *N. crassa* and *S. fimicola*. Some non-conserved regions were predicted in *N. crassa* on Serine and Threonine (S-91, T-297) and (S-173, T-322) in all strains of *S. fimicola*.

During the present investigation, we have found that Ser/Thr phosphatases (PKC and UNSP) are actively involved in the phosphorylation of the dipeptidyl-aminopeptidase in different strains of *S. fimicola* and *N.crassa*. PKC in fungi perform many important functions like regulation of cell, growth, synthesis of proteins and maintenance of cell integrity (Albataineh et al., 2014; Kosti et al., 2010). Serine/threonine phosphorylation modifications are important for the regulation of many cellular functions. We found many sites conserved by comparing the phosphorylation sites of both strains. These sites were defined on Serine (S-5, S-100) between *N. crassa* and *S. fimicola* strain S1. The conserved regions were defined on serine between *N. Crassa* and *S. fimicola*'s strain S2, S3 (S-23,S-91, S-94, S-96, S-155, S-175, S-177) and conserved regions on Threonine (T-589) have been detected between *N. crassa* and *S. fimicola* strain S1.The sites (S-7, S-14,T-11,T-63,T-212,T-274,T-924) of S. *fimicola* are different from N.crassa due to the genetic variation. The sites (S-8, T- 12, T-239, Y-12, Y-56, and Y-786) of *N. crassa* are different from *S. fimicola* due to genetic variation. In the current study, Phosphorylation of protease dipeptidyl aminopeptidases gene was observed on S-32 in S1 strain of *S. fimicola* which is also studied (Madzharova et al., 2019) in matrix metalloproteinases (MMPs) in humans. PTMs are affected by genetic variations. These genetic variations showed diverse protein motifs by PTMs which are necessary for the lives of organisms in adverse climates (Arif et al., 2017). The polymorphism can also be observed in the specific gene regions of nucleotide sequences within species and closely related organisms (Park et al., 2007). Same strategies to predict PTMs of histone 3 and 4 protein, manganese oxide super disumtase protein were adopted (Jawaria et al., 2018; Jamil et al., 2018). Nuclear export possesses several pathways (Ossareh-Nazari et al., 2001). The best nuclear export pathway is a Leucine-rich nuclear export signal (NES). La Cour and his colleagues said that nuclear export signals help the factors and proteins to interact with them to leave the cytoplasm. Nuclear

export signals on residue 250-L and 275-M in *N.crassa* and *S. fimicola* have been predicted (La Cour et al., 2004). The prediction of NES at position 328 of frequency clock protein in *S. fimicola* and 323 in *N. crassa* was reported by (Bukhari et al., 2020).

Similarly the present results show change (mutation) in genetic makeup of *S. fimicola* strains due to which a great difference is seen in protease enzyme activity and its biomass production as south strain (S2) showed more enzyme activity and biomass production under different physical parameters of growth than north strain (N6) that is due to change in nucleotide sequences and amino acid sequences of both side strains and it is concluded that South facing slop had harsh conditions for *S. fimicola* growth which mutate its genetic makeup and increase the production of protease enzyme in S2 strain (Daly et al., 2020; Toghueo et al., 2021). 3D models of protease dipeptidyl aminopeptidase have been visualized in PyMOL. Sticks represent coil structure, arrows represent β - sheet and sticks and cartoon coils indicate α - helix. (a). *N. crassa* with dimensions (Å):X:89.158 Y:70.858 Z:86.334. (b) *S. fimicola* S1 having dimensions (Å):X:89.158 Y:70.085 Z:86.043. (c). *S. fimicola* N5 showing dimensions (Å): X:89.158 Y:69.975 Z:86.334 in Figure 5.

5. Conclusion

This research helped us to explore the genes involved in the production of proteases of *S. fimicola*. Proteases occupy an important position and play a pivotal role in dealing with commercially important enzymes. Microbial proteases are commonly utilized in several industries such as brewing, detergent, leather, dairy, and food-processing. This study helped to find out the genetic variations among different strains of *S. fimicola* due to environmental stress and helped in establishing the basis and platform for the production of proteases on small as well as on large scale.

References

ABIDI, F., LIMAM, F. and NEJIB, M.M., 2008. Production of alkaline proteases by *Botrytis cinerea* using economic raw materials: assay as biodetergent. *Process Biochemistry*, vol. 43, no. 11, pp. 1202-1208. http://dx.doi.org/10.1016/j.procbio.2008.06.018.

ALBATAINEH, M.T., LAZZELL, A., LOPEZ-RIBOT, J.L. and KADOSH, D., 2014. Ppg1, a PP2A-type protein phosphatase, controls filament extension and virulence in *Candida albicans*. *Eukaryotic Cell*, vol. 13, no. 12, pp. 1538-1547. http://dx.doi.org/10.1128/EC.00199-14. PMid:25326520.

ANNA-ARRIOLA, S.S. and HERSKOWITZ, I., 1994. Yeast sequencing reports. Isolation and DNA sequence of the STE13 gene encoding dipeptidyl aminopeptidase. *Yeast*, vol. 10, no. 6, pp. 801-810. http://dx.doi.org/10.1002/yea.320100610. PMid:7975897.

ARIF, R., AKRAM, F., JAMIL, T., MUKHTAR, H., LEE, S.F. and SALEEM, M., 2017. Genetic variation and its reflection on posttranslational modifications in frequency clock and mating type a-1 proteins in Sordaria fimicola. *BioMed Research International*, vol. 2017, pp. 1-10. http://dx.doi.org/10.1155/2017/1268623.

ARNAU, J., YAVER, D. and HJORT, C.M., 2020. Strategies and challenges for the development of industrial enzymes using fungal cell factories. In: H. NEVALAINEN, ed. *Grand challenges in fungal biotechnology*. Cham: Springer, pp. 179-210. http://dx.doi.org/10.1007/978-3-030-29541-7_7.

BENMRAD, M.O., MECHRI, S., ZARAÎ JAOUADI, N., BEN ELHOUL, M., REKIK, H., SAYADI, S., BEJAR, S., KECHAOU, N. and JAOUADI, B., 2019. Purification and biochemical characterization of a novel thermostable protease from the oyster mushroom Pleurotus sajor-caju strain CTM10057 with industrial interest. *BMC Biotechnology*, vol. 19, no. 1, pp. 1-18. http://dx.doi.org/10.1186/s12896-019-0536-4. PMid:30606176.

BUKHARI, S.H., MOBEEN, I., NAUREEN, U., AKRAM, F., ARIF, R., SHAHID, M.G. and SALEEM, M., 2020. Analysis of genetic polymorphisms and post translational modifications of cytochrome C-1 in *Sordaria fimicola*. *International Journal of Agriculture and Biology*, vol. 23, no. 3, pp. 675-680.

BUNNIK, E.M. and LE ROCH, K.G., 2013. An introduction to functional genomics and systems biology. *Advances in Wound Care*, vol. 2, no. 9, pp. 490-498. http://dx.doi.org/10.1089/wound.2012.0379. PMid:24527360.

CUPP-ENYARD, C., 2008. Sigma's non-specific protease activity assay-casein as a substrate. *Journal of Visualized Experiments*, vol. 19, no. 19, pp. e899. http://dx.doi.org/10.3791/899.

DALY, P., PENG, M., MITCHELL, H.D., KIM, Y.M., ANSONG, C., BREWER, H., DE GIJSEL, P., LIPTON, M.S., MARKILLIE, L.M., NICORA, C.D., ORR, G., WIEBENGA, A., HILDÉN, K.S., KABEL, M.A., BAKER, S.E., MÄKELÄ, M.R. and DE VRIES, R.P., 2020. Colonies of the fungus *Aspergillus niger* are highly differentiated to adapt to local carbon source variation. *Environmental Microbiology*, vol. 22, no. 3, pp. 1154-1166. http://dx.doi.org/10.1111/1462-2920.14907. PMid:31876091.

DE VRIES, R.P., PATYSHAKULIYEVA, A., GARRIGUES, S. and AGARWAL-JANS, S., 2020. The current biotechnological status and potential of plant and algal biomass degrading/modifying enzymes from Ascomycete Fungi. In: H. NEVALAINEN, ed. *Grand challenges in fungal biotechnology*. Cham: Springer, pp. 81-120. http://dx.doi.org/10.1007/978-3-030-29541-7_4.

DIAS, D.R., VILELA, D.M., SILVESTRE, M.P.C. and SCHWAN, R.F., 2008. Alkaline protease from *Bacillus sp.* isolated from coffee bean grown on cheese whey. *World Journal of Microbiology & Biotechnology*, vol. 24, no. 10, pp. 2027-2034. http://dx.doi.org/10.1007/s11274-008-9706-6.

DTU HEALTH TECH, 2021a [viewed 25 August 2021]. *YinOYang - 1.2* [online]. Available from: http://www.cbs.dtu.dk/services/YinOYang/

DTU HEALTH TECH, 2021b [viewed 25 August 2021]. *NetPhos - 3.1* [online]. Available from: http://www.cbs.dtu.dk/services/NetPhos//

EMBOSS TRANSEQ [online], 2021 [viewed 25 August 2021]. Available from: https://www.ebi.ac.uk/Tools/st/emboss_transeq/

FERRERO, M.A., CASTRO, G., ABATE, C., BAIGORI, M. and SIÑERIZ, F., 1996. Thermostable alkaline proteases of Bacillus licheniformis MIR 29: isolation, production and characterization. *Applied Microbiology and Biotechnology*, vol. 45, no. 3, pp. 327-332. http://dx.doi.org/10.1007/s002530050691.

HANKIN, L. and ANAGNOSTAKIS, S., 1975. The use of solid media for detection of enzyme production by fungi. *Mycologia*, vol. 67, no. 3, pp. 597-607. http://dx.doi.org/10.1080/00275514.1975.12019782.

HOFMANN, S.L., ATASHBAND, A., CHO, S.K., DAS, A.K., GUPTA, P. and LU, J.Y., 2002. Neuronal ceroid lipofuscinoses caused by defects in soluble lysosomal enzymes (CLN1 and CLN2). *Current*

Molecular Medicine, vol. 2, no. 5, pp. 423-437. http://dx.doi.org/10.2174/1566524023362294. PMid:12125808.

HU, G. and LEGER, R.J., 2004. A phylogenomic approach to reconstructing the diversification of serine proteases in fungi. *Journal of Evolutionary Biology*, vol. 17, no. 6, pp. 1204-1214. http://dx.doi.org/10.1111/j.1420-9101.2004.00786.x. PMid:15525405.

ISHFAQ, M., MAHMOOD, N., NASIR, I.A. and SALEEM, M., 2017. Biochemical and molecular analysis of laccase enzyme in Saprobic Fungus; S*ordaria fimicola. International Journal of Agriculture and Biology*, vol. 19, no. 1, pp. 204-210. http://dx.doi.org/10.17957/IJAB/15.0265.

JAMIL, T., SAMI, N., ARIF, R., RASHID, Q. and SALEEM, M., 2018. H3/H4 histone genes variations and its effect on post translational modifications in various strains of Sordaria fimicola. *International Journal of Agriculture and Biology*, vol. 20, no. 5, pp. 1021-1026.

JAWARIA, R., RABIA, A., MUHAMMAD, I., LEE, S. and SALEEM, M., 2018. Amplification and posttranslational modification study of Manganese Superoxide dismutase gene in sordaria fimicola. *International Journal of Agriculture and Biology*, vol. 20, no. 9, pp. 2059-2063.

JENSEN, B., NEBELONG, P., OLSEN, J. and REESLEV, M., 2002. Enzyme production in continuous cultivation by the thermophilic fungus, *Thermomyces lanuginosus*. *Biotechnology Letters*, vol. 24, no. 1, pp. 41-45. http://dx.doi.org/10.1023/A:1013805232462.

KALISZ, H.M., 1988. Microbial proteinases. *Advances in Biochemical Engineering/Biotechnology*, vol. 36, pp. 1-65. http://dx.doi.org/10.1007/BFb0047944. PMid:3142224.

KARTHIC, J., SIDDALINGESHWARA, K., SUNILDUTT, P., PRAMOD, T. and VISHWANATHA, T., 2014. An approach isolation, screening and production of protease from *Aspergillus oryzae*. *Journal of Drug Delivery and Therapeutics*, vol. 4, no. 2, pp. 86-89. http://dx.doi.org/10.22270/jddt.v4i2.816.

KHENG, P.P. and OMAR, I.C., 2005. Xylanase production by a local fungal isolate, Aspergillus niger USM AI 1 via solid state fermentation using palm kernel cake (PKC) as substrate. *Songklanakarin Journal of Science and Technology*, vol. 27, no. 2, pp. 325-336.

KOSTI, I., MANDEL-GUTFREUND, Y., GLASER, F. and HORWITZ, B.A., 2010. Comparative analysis of fungal protein kinases and associated domains. *BMC Genomics*, vol. 11, no. 1, pp. 133. http://dx.doi.org/10.1186/1471-2164-11-133. PMid:20178650.

KRISHNA, V.K., GUPTA, M., GUPTA, N., GAUDANI, H., GUPTA, N., GUPTA, M. and MISHRA, D., 2009. Optimization of growth and production of protease by *Penicillium* species using submerged fermentation. *International Journal of Microbiology Research*, vol. 1, no. 1, pp. 14-18. http://dx.doi.org/10.9735/0975-5276.1.1.14-18.

KUMAR, A.G., NAGESH, N., PRABHAKAR, T. and SEKARAN, G., 2008. Purification of extracellular acid protease and analysis of fermentation metabolites by *Synergistes* sp. utilizing proteinaceous solid waste from tanneries. *Bioresource Technology*, vol. 99, no. 7, pp. 2364-2372. http://dx.doi.org/10.1016/j.biortech.2007.05.001. PMid:17601727.

LA COUR, T., KIEMER, L., MØLGAARD, A., GUPTA, R., SKRIVER, K. and BRUNAK, S., 2004. Analysis and prediction of leucine-rich nuclear export signals. *Protein Engineering, Design & Selection*, vol. 17, no. 6, pp. 527-536. http://dx.doi.org/10.1093/protein/gzh062. PMid:15314210.

LANGE, L., AGGER, J.W. and MEYER, A.S., 2020. Fungal biotechnology: unlocking the full potential of fungi for a more sustainable world. In: H. NEVALAINEN, ed. *Grand challenges in fungal biotechnology*. Cham: Springer, pp. 3-32. http://dx.doi.org/10.1007/978-3-030-29541-7_1.

LEE, H.S., QI, Y. and IM, W., 2015. Effects of N-glycosylation on protein conformation and dynamics: protein Data Bank analysis and molecular dynamics simulation study. *Scientific Reports*, vol. 5, no. 1, pp. 1-7.

MA, Z. and LEE, Y., 2008. Patent application and technological collaboration in inventive activities: 1980–2005. *Technovation*, vol. 28, no. 6, pp. 379-390. http://dx.doi.org/10.1016/j.technovation.2007.07.011.

MADZHAROVA, E., KASTL, P., SABINO, F. and AUF DEM KELLER, U., 2019. Post-translational modification-dependent activity of matrix metalloproteinases. *International Journal of Molecular Sciences*, vol. 20, no. 12, pp. 3077. http://dx.doi.org/10.3390/ijms20123077. PMid:31238509.

MCCLUSKEY, K., WIEST, A. and PLAMANN, M., 2010. The Fungal Genetics Stock Center: a repository for 50 years of fungal genetics research. *Journal of Biosciences*, vol. 35, no. 1, pp. 119-126. http://dx.doi.org/10.1007/s12038-010-0014-6. PMid:20413916.

MEHROTRA, S., PANDEY, P., GAUR, R. and DARMWAL, N., 1999. The production of alkaline protease by a Bacillus species isolate. *Bioresource Technology*, vol. 67, no. 2, pp. 201-203. http://dx.doi.org/10.1016/S0960-8524(98)00107-2.

MEHTA, V., THUMAR, J. and SINGH, S., 2006. Production of alkaline protease from an alkaliphilic actinomycete. *Bioresource Technology*, vol. 97, no. 14, pp. 1650-1654. http://dx.doi.org/10.1016/j.biortech.2005.07.023. PMid:16203132.

MOHAPATRA, B., BAPUJI, M. and SREE, A., 2003. Production of industrial enzymes (amylase, carboxymethylcellulase and protease) by bacteria isolated from marine sedentary organisms. *Acta Biotechnologica*, vol. 23, no. 1, pp. 75-84. http://dx.doi.org/10.1002/abio.200390011.

MORIMURA, S., KIDA, K. and SONODA, Y., 1994. Production of protease using wastewater from the manufacture of shochu. *Journal of Fermentation and Bioengineering*, vol. 77, no. 2, pp. 183-187. http://dx.doi.org/10.1016/0922-338X(94)90321-2.

MUSZEWSKA, A., STEPNIEWSKA-DZIUBINSKA, M.M., STECZKIEWICZ, K., PAWLOWSKA, J., DZIEDZIC, A. and GINALSKI, K., 2017. Fungal lifestyle reflected in serine protease repertoire. *Scientific Reports*, vol. 7, no. 1, pp. 9147. http://dx.doi.org/10.1038/s41598-017-09644-w. PMid:28831173.

NASCIMENTO, W.C.A.D. and MARTINS, M.L.L., 2004. Production and properties of an extracellular protease from thermophilic Bacillus sp. *Brazilian Journal of Microbiology*, vol. 35, no. 1-2, pp. 91-96. http://dx.doi.org/10.1590/S1517-83822004000100015.

NOWROUSIAN, M., STAJICH, J.E., CHU, M., ENGH, I., ESPAGNE, E., HALLIDAY, K., KAMEREWERD, J., KEMPKEN, F., KNAB, B., KUO, H.C., OSIEWACZ, H.D., PÖGGELER, S., READ, N.D., SEILER, S., SMITH, K.M., ZICKLER, D., KÜCK, U. and FREITAG, M., 2010. De novo assembly of a 40 Mb eukaryotic genome from short sequence reads: sordaria macrospora, a model organism for fungal morphogenesis. *PLOS Genetics*, vol. 6, no. 4, pp. e1000891. http://dx.doi.org/10.1371/journal.pgen.1000891. PMid:20386741.

OSSAREH-NAZARI, B., GWIZDEK, C. and DARGEMONT, C., 2001. Protein export from the nucleus. *Traffic*, vol. 2, no. 10, pp. 684-689. http://dx.doi.org/10.1034/j.1600-0854.2001.21002.x. PMid:11576444.

PARK, E.-J., FUKUDA, S., ENDO, H., KITADE, Y. and SAGA, N., 2007. Genetic polymorphism within Porphyra yezoensis (Bangiales, Rhodophyta) and related species from Japan and Korea detected by cleaved amplified polymorphic sequence analysis. *European Journal of Phycology*, vol. 42, no. 1, pp. 29-40. http://dx.doi.org/10.1080/09670260601127681.

PENG, Y., HUANG, Q., ZHANG, R.-H. and ZHANG, Y.Z., 2003. Purification and characterization of a fibrinolytic enzyme

produced by *Bacillus amyloliquefaciens* DC-4 screened from *douchi*, a traditional Chinese soybean food. *Comparative Biochemistry and Physiology. Part B, Biochemistry & Molecular Biology*, vol. 134, no. 1, pp. 45-52. http://dx.doi.org/10.1016/S1096-4959(02)00183-5. PMid:12524032.

PINHO, S.S. and REIS, C.A., 2015. Glycosylation in cancer: mechanisms and clinical implications. *Nature Reviews. Cancer*, vol. 15, no. 9, pp. 540-555. http://dx.doi.org/10.1038/nrc3982. PMid:26289314.

RAO, M.B., TANKSALE, A.M., GHATGE, M.S. and DESHPANDE, V.V., 1998. Molecular and biotechnological aspects of microbial proteases. *Microbiology and Molecular Biology Reviews*, vol. 62, no. 3, pp. 597-635. http://dx.doi.org/10.1128/MMBR.62.3.597-635.1998. PMid:9729602.

RAZZAQ, A., SHAMSI, S., ALI, A., ALI, Q., SAJJAD, M., MALIK, A. and ASHRAF, M., 2019. Microbial proteases applications. *Frontiers in Bioengineering and Biotechnology*, vol. 7, pp. 110. http://dx.doi.org/10.3389/fbioe.2019.00110. PMid:31263696.

SABU, A., SARITA, S., PANDEY, A., BOGAR, B., SZAKACS, G. and SOCCOL, C.R., 2002. Solid-state fermentation for production of phytase by Rhizopus oligosporus. *Applied Biochemistry and Biotechnology*, vol. 102-103, no. 1-6, pp. 251-260. http://dx.doi.org/10.1385/ABAB:102-103:1-6:251. PMid:12396128.

SAGHAI-MAROOF, M.A., SOLIMAN, K.M., JORGENSEN, R.A. and ALLARD, R.W., 1984. Ribosomal DNA spacer-length polymorphisms in barley: mendelian inheritance, chromosomal location, and population dynamics. *Proceedings of the National Academy of Sciences of the United States of America*, vol. 81, no. 24, pp. 8014-8018. http://dx.doi.org/10.1073/pnas.81.24.8014. PMid:6096873.

SAMARNTARN, W., CHEEVADHANARAK, S. and TANTICHAROEN, M., 1999. Production of alkaline protease by a genetically engineered Aspergillus oryzae U1521. *The Journal of General and Applied Microbiology*, vol. 45, no. 3, pp. 99-103. http://dx.doi.org/10.2323/jgam.45.99. PMid:12501379.

SANTHOSE, I., GNANADOSS, J. and SELVARAJAN, R. and ELUMALAI, S., 2011. Enhanced carotenoid synthesis of *Phormidium* sp. in stressed conditions. *Journal of Experimental Sciences*, vol. 2, no. 3, pp. 38-44.

SATHYA, R., PRADEEP, B., ANGAYARKANNI, J. and PALANISWAMY, M., 2009. Production of milk clotting protease by a local isolate of *Mucor circinelloides* under SSF using agro-industrial wastes. *Biotechnology and Bioprocess Engineering; BBE*, vol. 14, no. 6, pp. 788-794. http://dx.doi.org/10.1007/s12257-008-0304-0.

SINDHU, R., SUPRABHA, G. and SHASHIDHAR, S., 2009. Optimization of process parameters for the production of alkaline protease from Penicillium godlewskii SBSS 25 and its application in detergent industry. *African Journal of Microbiological Research*, vol. 3, no. 9, pp. 515-522.

SOOKKHEO, B., SINCHAIKUL, S., PHUTRAKUL, S. and CHEN, S.T., 2000. Purification and characterization of the highly thermostable proteases from Bacillus stearothermophilus TLS33. *Protein Expression and Purification*, vol. 20, no. 2, pp. 142-151. http://dx.doi.org/10.1006/prep.2000.1282. PMid:11049738.

TÉLLEZ-TÉLLEZ, M. and DÍAZ-GODÍNEZ, G., 2019. Omic tools to study enzyme production from fungi in the *Pleurotus genus. BioResources*, vol. 14, no. 1, pp. 2420-2457. http://dx.doi.org/10.15376/biores.14.1.2420-2457.

TOGHUEO, R.M.K., KEMGNE, E.A.M., SAHAL, D., YADAV, M., KENOU KAGHO, D.U., YANG, B., BAKER, B.J. and BOYOM, F.F., 2021. Specialized antiplasmodial secondary metabolites from *Aspergillus niger* 58, an endophytic fungus from *Terminalia catappa*. *Journal of Ethnopharmacology*, vol. 269, pp. 113672. http://dx.doi.org/10.1016/j.jep.2020.113672. PMid:33301916.

VINOGRADOVA, K., VLASOVA, I., SHARKOVA, T., DODZIN, M. and MAKSIMOV, V.N., 2003. L-glutamate oxidase from *Streptomyces cremeus* 510 MGU: effect of nitrogen sources on enzyme secretion. *Antibiotics and Chemotherapy*, vol. 48, no. 2, pp. 3-8.

VISHWANATHA, K.S., RAO, A.A. and SINGH, S.A., 2010. Production and characterization of a milk-clotting enzyme from *Aspergillus oryzae* MTCC 5341. *Applied Microbiology and Biotechnology*, vol. 85, no. 6, pp. 1849-1859. http://dx.doi.org/10.1007/s00253-009-2197-z. PMid:19727708.